

# Complement depletion protects lupus-prone mice from ischemia-reperfusion-initiated organ injury

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**Ioannou A, Lieberman LA, Dalle Lucca JJ, Tsokos GC.** Complement depletion protects lupus-prone mice from ischemia-reperfusion-initiated organ injury. *Am J Physiol Gastrointest Liver Physiol* 304: G283–G292, 2013. First published October 25, 2012; doi:10.1152/ajpgi.00371.2012.—Ischemia-reperfusion (IR) injury causes a vigorous immune response that is amplified by complement activation, leading to local and remote tissue damage. Using MRL/*lpr* mice, which are known to experience accelerated tissue damage after mesenteric IR injury, we sought to evaluate whether complement inhibition mitigates organ damage. We found that complement depletion with cobra venom factor protected mice from local and remote lung tissue damage. Protection from injury was associated with less complement (C3) and membrane attack complex deposition, less neutrophil infiltration, and lower levels of local proinflammatory cytokine production. In addition, complement depletion was able to decrease the level of oxidative stress as measured by glutathione peroxidase 1 mRNA levels and superoxide dismutase activity. Furthermore, blockage of C5a receptor protected MRL/*lpr* mice from local tissue damage, but not from remote lung tissue damage. In conclusion, although treatments with cobra venom factor and C5a receptor antagonist were able to protect mice from local tissue damage, treatment with C5a receptor antagonist was not able to protect mice from remote lung tissue damage, implying that more factors contribute to the development of remote tissue damage after IR injury. These data also suggest that complement inhibition at earlier, rather than late, stages can have clinical benefit in conditions that are complicated with IR injury.

ischemia-reperfusion injury; intestine; complement; systemic lupus erythematosus

MESENTERIC ISCHEMIA-REPERFUSION (IR) injury is encountered after restoration of intestinal blood supply following a brief period of ischemia. The reestablishment of blood flow leads to a robust activation of the innate and adaptive immune response (22, 44, 14), which results in local organ injury and, subsequently, damage of remote organs. Production of reactive oxygen species (3, 27) and deposition of natural antibodies on newly expressed antigens on the ischemic cells (28, 40) are the main initiators of this cascade. The extensive activation of the complement cascade contributes to the amplification of the immune response, which eventually leads to vascular injury, increased mucosal permeability, and tissue necrosis (5, 6, 19, 42).

The complement system consists of proteins that are synthesized primarily by the liver and are found in the blood

as inactive precursors. The complement system is activated through three different cascades, the classical, alternative, and lectin pathways, and is an important mediator of innate immune defense and inflammation (50, 51). Complement proteins are activated through protease cleavage, resulting in a significant increase in bioactive complement fragments such as C3a and C5a (50, 51). Although various studies using C3-deficient mice (43), factor B-deficient mice (21), and mannose-binding lectin (MBL)-deficient mice (34, 52) showed the importance of all three pathways to the expression of tissue damage after IR injury, more studies are needed to elucidate the exact mechanisms whereby complement orchestrates the immune response.

Understanding the mechanisms involved in the tissue damage that follows IR is important in improving the clinical outcome of life-threatening conditions such as trauma/hemorrhagic shock (41), organ transplantation (8), and cardiovascular diseases (46). IR injury may also affect the disease process in patients with autoimmune diseases such as systemic lupus erythematosus (SLE) (1, 36, 47). SLE is characterized by the production of autoantibodies directed against a variety of self-antigens and the formation of immune complexes (45). Circulating autoantibodies and immune complexes are deposited on vessel walls and activate complement, which leads to local inflammation and vasculopathies, which, in turn, lead to accelerated atherosclerosis with coronary heart disease (22) and gastrointestinal small vessel vasculitis (39). The combination of thrombotic events and various vasculopathies could be responsible for an increased incidence of transient episodes of ischemia and reperfusion in SLE patients. However, in a disease with a high level of complexity, such as SLE, more studies are needed to clarify the importance of IR injury in the overall pathophysiology of the disease. In support of this concept are data that have shown that the lupus-prone mouse B6.MRL/*lpr* displays increased and accelerated organ damage after mesenteric IR and that the injury depends on the presence of autoantibodies and the subsequent complement activation (15, 54).

Complement depletion or blockage of the complement pathway using molecules such as cobra venom factor (CVF) (24, 33) and C5a receptor antagonists (C5aRA) (49) has been demonstrated to protect from tissue damage in various IR animal models. However, the effect of complement depletion or complement receptor blockage in the context of increased levels of autoantibodies and continuous complement activation has not been evaluated. In this study, we show that complement depletion protects lupus-prone mice from local and remote IR injury and that blockage of C5a

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receptors protects mice from local tissue damage, but not from remote lung damage.

## MATERIALS AND METHODS

**Mice.** Adult (12- to 14-wk-old) female MRL/*lpr* mice (Jackson Laboratory, Bar Harbor, ME) were used for all experiments. All mice were maintained in specific pathogen-free conditions in the animal research facility at the Beth Israel Deaconess Medical Center. All experiments were performed in accordance with the guidelines and approval of the Institutional Animal Care and Use Committee of the Beth Israel Deaconess Medical Center.

**IR injury procedure.** Mice were randomly assigned to sham or IR groups. Mice were anesthetized by injection of tribromoethanol (Avertin, 250 mg/kg ip) and maintained under anesthesia with Avertin (125 mg/kg ip). The procedure was done as previously described (56). Briefly, a midline laparotomy was performed, and the superior mesenteric artery was isolated and clamped with a small microvascular clip for 30 min. The clip was removed, and the intestine was reperfused for 2 h. Sham-treated mice underwent the same surgical procedure without artery clamping. The incision was sutured with 4.0 SOFASILK (Synture, Mansfield, MA), and the mice were resuscitated with subcutaneous administration of 1.0 ml of prewarmed sterile PBS and monitored during the reperfusion period. Body temperature was maintained at 37°C throughout the experimental procedure. At the end of the reperfusion period, mice were euthanized by carbon dioxide asphyxiation. Mice were injected intraperitoneally with a total of 24 U of CVF in 0.5 ml of PBS at 24 and 16 h before the surgery. Control mice were injected intraperitoneally with the same volume of PBS. A specific C5aRA (PMX53, 1 mg/kg body wt in PBS), the cyclic hexapeptide Ac-Phe-[Orn-Pro-dCha-Trp-Arg], was used. After 30 min of ischemia, the mice were injected intraperitoneally with the peptide. Control mice were injected with the control peptide (Ac-Phe-[Orn-Pro-dCha-Ala-dArg]) at the same concentration.

**Histology.** Histopathology was determined on formalin-fixed paraffin-embedded 6- to 8- $\mu$ m sections stained with hematoxylin and eosin and examined using a light microscope.

For intestinal villi injury scoring, 50–100 villi per tissue section were graded as previously described (7): 0 for a normal-appearing

villus, 1 for villi demonstrating tip distortion, 2 for villi without goblet cells and with Guggenheim's spaces, 3 for villi exhibiting patchy disruption of the epithelial cells, 4 for villi demonstrating exposed, intact lamina propria and sloughing of epithelial cells, 5 for villi demonstrating exuding lamina propria, and 6 for villi with hemorrhage or denudation. In the case of lungs, alveolar and periluminal injury scores for each lung section were calculated on the basis of the method of Cooke et al. (10). Ten fields at  $\times 400$  magnification were viewed for each lung section and scored for alveolar infiltration as follows: 0 when no infiltrate was present, 1 when the infiltrate could be visualized easily only at  $\times 400$  magnification, 2 when infiltrates were readily visible, and 3 for consolidation. Similarly, each section was scored for periluminal damage (airway or blood vessel) at  $\times 100$  magnification as follows: 0 when there was no infiltrate, 1 when the infiltrate was 1–3 cell layers thick, 2 when the infiltrate was 4–10 cell layers thick, and 3 when the infiltrate was  $>10$  cell layers thick. On the basis of the overall involvement of the section, a severity score was calculated as follows: 1 for 0–25% involvement, 2 for 25–50% involvement, and 3 for  $>50\%$  involvement. For calculation of the total lung injury score, the means of alveolar and periluminal scores for each section were summed and multiplied by the severity score, which gave a final score of 0–18.

**Immunohistochemical staining of paraffin-embedded tissues.** Formalin-fixed paraffin sections of small intestine were subjected to rehydration, and endogenous peroxidase activity was quenched by 3%  $H_2O_2$ . Then antigen retrieval was performed using Retrieval A (BD Pharmingen, San Jose, CA) according to the manufacturer's directions. The sections were blocked with 10% BSA-PBS containing the serum from host species of secondary antibody. Primary antibodies prepared in 10% BSA-PBS were applied overnight at 4°C. For immunohistochemical (IHC) studies, the following reagents were used: mouse anti-complement 3 (C3), rabbit anti-mouse C5b-C9 (ab55811, Abcam), horseradish peroxidase (HRP)-conjugated goat anti-rabbit IgG, HRP-conjugated goat anti-mouse IgG, and normal goat serum (Jackson ImmunoResearch, West Grove, PA). Consecutive tissue sections were stained with appropriate isotype controls. The slides were then incubated with HRP-conjugated secondary antibody for 60 min at room temperature, developed with NovaRED (Vector

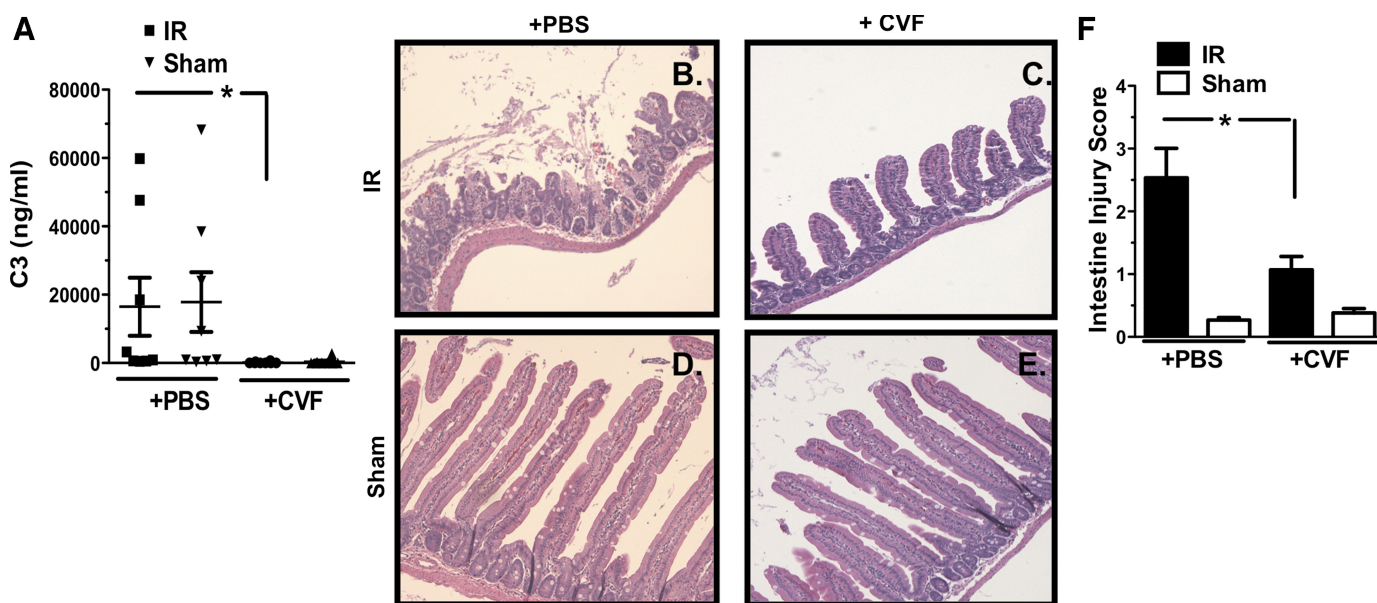


Fig. 1. Intestinal tissue damage is reduced following mesenteric ischemia-reperfusion (IR) in complement-depleted MRL/*lpr* mice. A: C3 complement factor levels as measured by ELISA ( $n = 8$  per group). CVF, cobra venom factor. B–E: hematoxylin-eosin-stained sections of small intestine from control (Sham) mice and mice exposed to 30 min of ischemia and 2 h of reperfusion. Representative images are shown at  $\times 100$  magnification. F: injury score in intestine. Values are means  $\pm$  SD of a total of 6 mice for each control and experimental group in 3 separate experiments.  $*P \leq 0.05$ .



Laboratories, Burlingame, CA), counterstained with hematoxylin, and dehydrated. The sections were mounted in mounting medium (Thermo Scientific, Waltham, MA).

For neutrophil staining, the fast blue salt method for detecting esterase reaction in neutrophils was performed. Briefly, formalin-fixed paraffin sections of small intestine were subjected to rehydration and incubated with chloroacetate solution [Naphesol AS-D (N-0758, Sigma) and fast blue BB salt (F-3378, Sigma)] for 1.5 h at room temperature in darkness. The slides were counterstained in freshly filtered Vector nuclear fast red for 5 min at room temperature and mounted in aqueous mounting medium (Fluoromount-G, Southern Biotech). All images were viewed and captured using a Nikon Eclipse 80i microscope.

**Real-time PCR protocol.** Real-time RT-PCR was performed with the LightCycler 480 system (Roche, South San Francisco, CA) using TaqMan gene expression master mix and predesigned TaqMan probes for mouse IL-6, IL-1 $\beta$ , TNF- $\alpha$ , glutathione peroxidase 1 (GPX-1), and GAPDH, as recommended by Applied Biosystems. The averaged cycle threshold ( $C_t$ ) values of each reaction derived from the target gene, determined with LightCycler 480 system software (Roche), were normalized to GAPDH levels.  $C_t$  values were used to calculate relative mRNA expression by the  $\Delta\Delta C_t$  relative quantification method.

**Antibodies and reagents.** CVF was purchased from Quidel (catalog no. A600). For measurement of C3 levels in mouse serum, the mouse C3 ELISA kit (catalog no. 130047, Genway) was used. For determination of superoxide dismutase activity, the superoxide dismutase

activity colorimetric assay kit (catalog no. ab65354, Abcam) was used according to the manufacturer's directions.

**Statistical analysis.** GraphPad Prism 4.0 for Windows (GraphPad Software, San Diego, CA) was used for all statistical calculations using the unpaired *t*-test. Values are means  $\pm$  SD. Differences were considered significant when  $P \leq 0.05$ .

## RESULTS

**CVF protects MRL/lpr mice from local tissue damage following mesenteric IR.** CVF forms a stable convertase with factor B and factor D that is resistant to hydrolysis and continuously activates C3 through the alternative complement pathway, eventually resulting in complement depletion (26). Because CVF has never been used in lupus-prone mice, we treated MRL/lpr mice with CVF and evaluated its ability to deplete complement. Mice received 12 U of CVF intraperitoneally in 0.5 ml of PBS at 24 and 16 h prior to mesenteric IR or sham procedure. Serum samples were obtained immediately after euthanasia, and the C3 levels were determined by ELISA. Mice treated with CVF did not have detectable levels of C3 compared the PBS-treated group (Fig. 1A). Next, we examined whether depletion of complement has a beneficial effect on expression of local intestinal damage. We compared the intestinal injury of complement-depleted mice with that of control

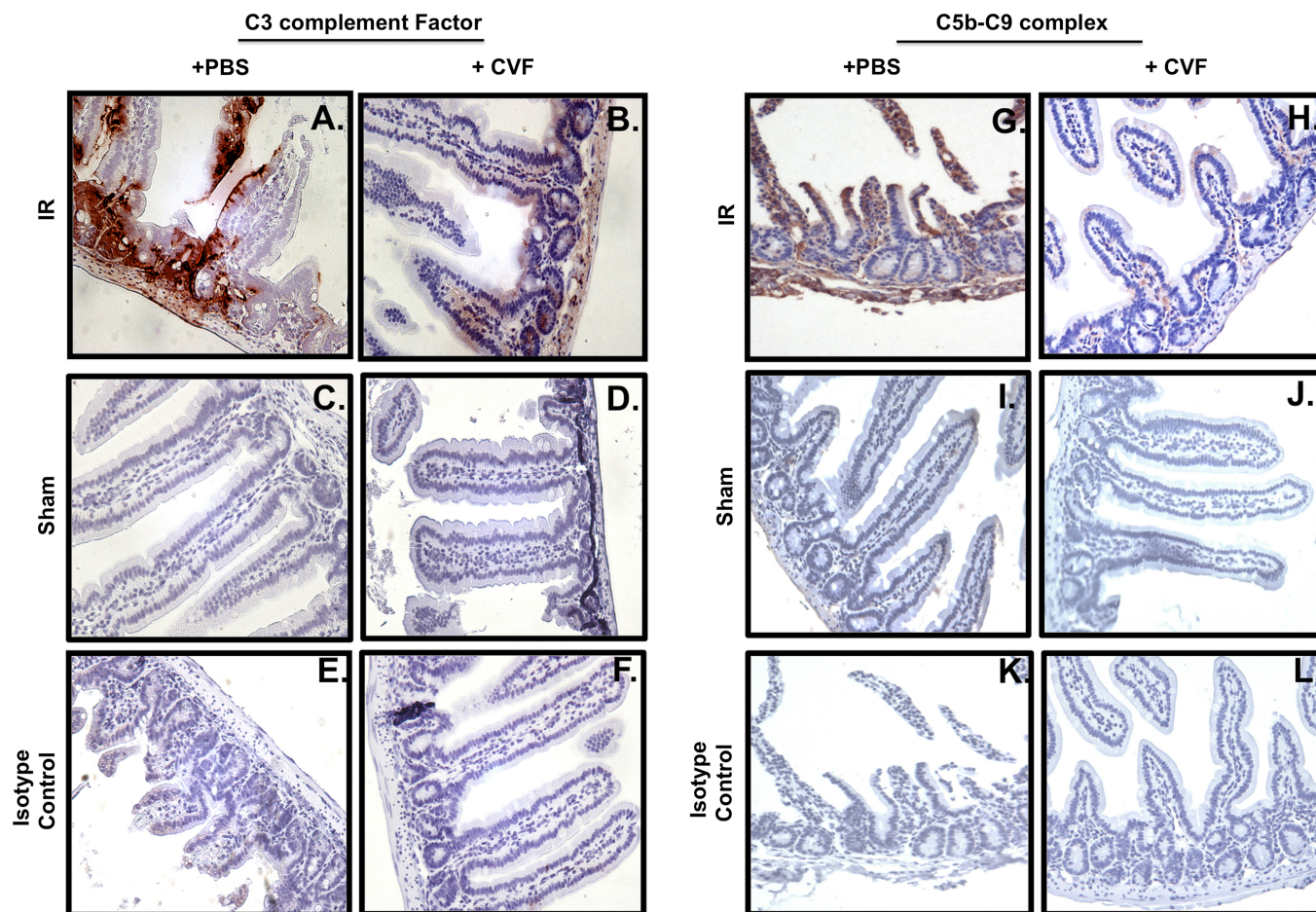


Fig. 2. C3 and C5b-C9 complex deposition in tissue is reduced following mesenteric IR in complement-depleted MRL/lpr mice. A–F: immunohistochemistry (IHC) with an anti-C3 antibody (red). G–L: IHC with an anti-C5b-C9 complex antibody (red). Representative images are shown at  $\times 100$  magnification. Nuclei stained blue with hematoxylin. A total of 6 mice were used for each control and experimental group in 3 separate experiments.



mice. Control mice subjected to IR displayed severe mucosal damage after 2 h of reperfusion compared with complement-depleted mice (Fig. 1, B–E). Decreased villus height, edema, and hemorrhage were prominent in the complement-sufficient mice. Cumulative data from three separate experiments are shown in Fig. 1F.

Although C3 levels were undetected in the serum of complement-depleted MRL/lpr mice, we sought to evaluate the level of local complement activation in the intestine by staining for C3 and C5b-9 complex deposition. We found a noticeable decrease in the deposition of C3 and C5b-9 in the intestine of complement-depleted compared with control mice, confirming that there was no local complement activation (Fig. 2).

**Complement depletion reduces local neutrophil infiltration and oxidative stress response following mesenteric IR.** It is well established that the increased production of reactive oxygen species and the increase in neutrophil infiltration in the ischemic tissue are important initiating events in the pathophysiology of IR injury (22). To evaluate whether complement is required in the evolution of these events, we stained intestinal tissues for neutrophils. We found reduced numbers of neutrophils in the intestine of complement-depleted compared with control mice (Fig. 3, A–D). To evaluate oxidative stress in the intestine, we measured mRNA levels of GPX-1, as well as the activity of superoxide dismutase, two very important en-

zymes in antioxidant defense. We found increased expression of GPX-1 in complement-depleted compared with control mice (Fig. 3E). Moreover, we observed a significant increase in superoxide dismutase activity in the complement-sufficient mice, which is consistent with an acute increase in oxidative stress in the intestine (Fig. 3F).

**Complement depletion decreases the mRNA levels of proinflammatory cytokines in MRL/lpr mice following mesenteric IR injury.** After ischemic injury, the levels of proinflammatory cytokines increase, indicating the presence of an inflammatory response. We measured the mRNA levels of the proinflammatory cytokines IL-6, IL-1 $\beta$ , and TNF- $\alpha$  to investigate the effect of complement depletion (Fig. 4). We observed significantly lower levels of proinflammatory cytokines in complement-depleted than control mice, and we can conclude that complement depletion can protect MRL/lpr mice from local tissue damage following mesenteric IR by decreasing the inflammatory response that leads to tissue damage.

**Complement depletion protects MRL/lpr mice from remote tissue damage following mesenteric IR.** As soon as the blood supply is reestablished at the ischemic tissue, proinflammatory cytokines and immune cells are transferred via the bloodstream to remote tissues such as the lung, where they are able to induce a secondary inflammatory response that can cause tissue damage (22). To further investigate the effect of complement depletion in remote lung tissue damage after mesenteric IR, we

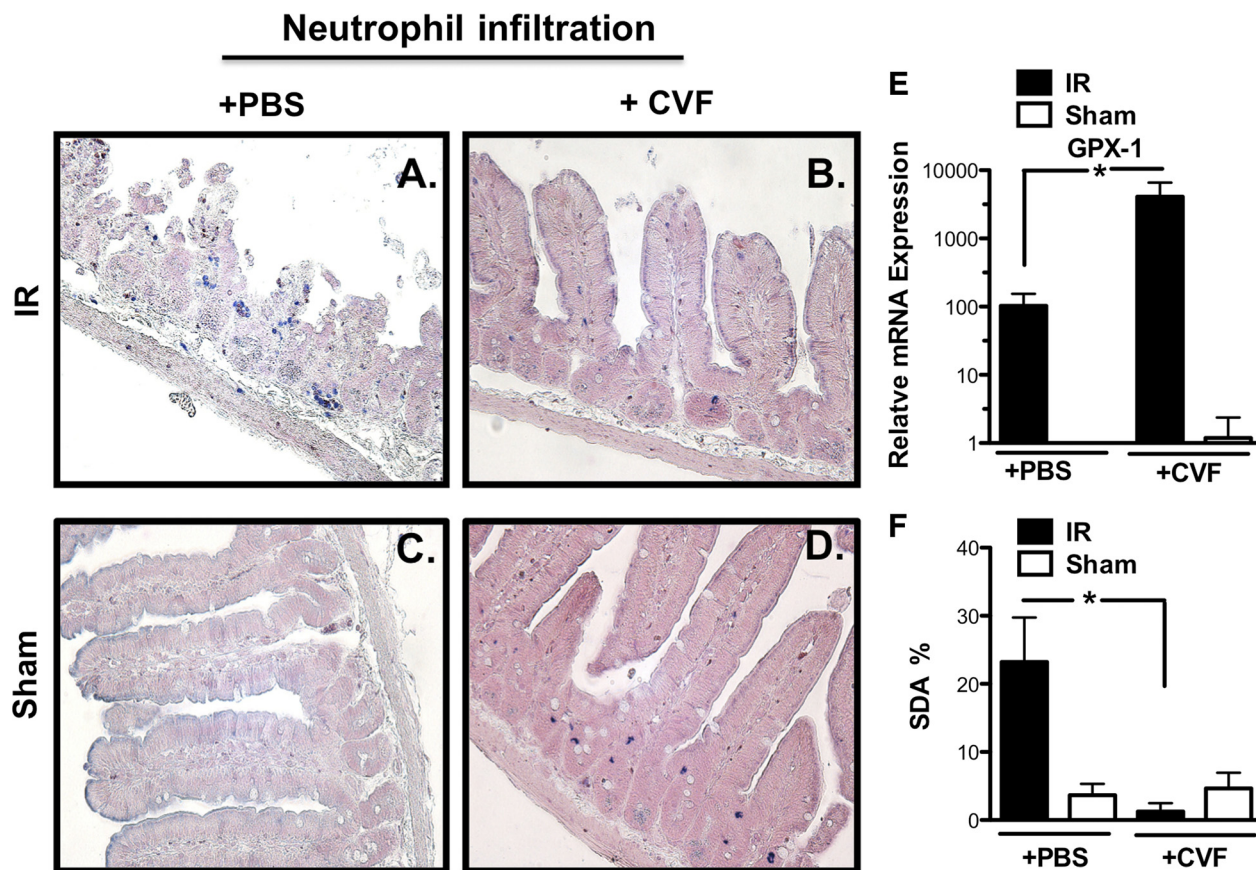


Fig. 3. Complement depletion reduces local neutrophil infiltration and oxidative stress in intestine of MRL/lpr mice following mesenteric IR. A–D: neutrophil infiltration evaluated using the fast blue salt method for detecting esterase reaction in neutrophils (blue). E: glutathione peroxidase 1 (GPX-1) mRNA levels after mesenteric IR injury in IR and Sham MRL/lpr mice. F: superoxide dismutase activity (SDA). All representative images are shown at  $\times 100$  magnification. A total of 6 mice were used for each control and experimental group in 3 separate experiments. \* $P < 0.05$ .

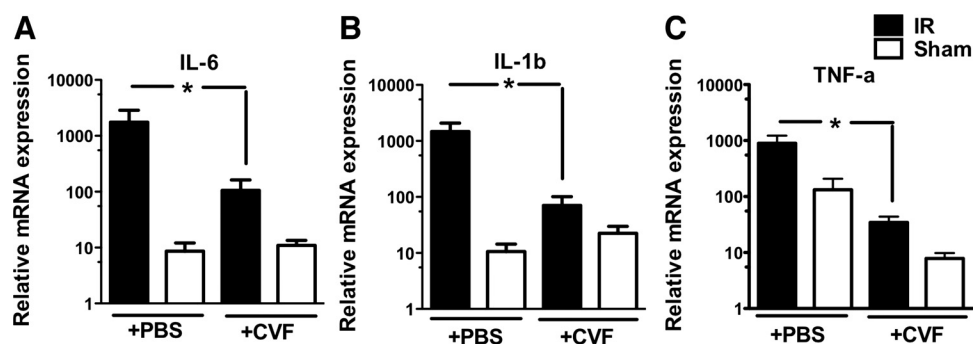


Fig. 4. Complement depletion decreases mRNA levels of proinflammatory cytokines in MRL/lpr mice following mesenteric IR. A–C: IL-6, IL-1 $\beta$ , and TNF- $\alpha$  mRNA levels in the intestine after mesenteric IR. mRNA expression was analyzed using quantitative RT-PCR. A total of 6 mice were used for each control and experimental group in separate experiments. \* $P \leq 0.05$ .

evaluated the levels of lung damage in MRL/lpr mice (Fig. 5). Complement-depleted mice displayed a significant reduction in lung damage (Fig. 5, A–D) compared with complement-sufficient MRL/lpr mice after mesenteric IR. Cumulative data in Fig. 5E show a statistically significant reduction in tissue damage in complement-depleted MRL/lpr compared with control mice. C3 deposition followed a pattern similar to that in the intestinal tissues (Fig. 6). Significantly less C3 deposition was observed in lung tissue from complement-depleted than control mice. These experiments indicate that complement activation can lead to remote tissue damage after mesenteric IR.

*C5aRA protects MRL/lpr mice from local, but not remote, tissue damage following mesenteric IR.* We showed that systemic depletion of complement leads to a reduction of local and remote lung tissue damage. However, long-term systemic complement depletion would most probably cause unwanted side effects, since complement activation has a very important role in

the immune response against infectious agents, as well as the clearance of immune complexes. To address this issue, we assessed whether blocking the effects of specific complement fragments after initiation of the complement cascade has protective effects on tissue damage. One of the most important complement fragments that has been implicated in the pathophysiology of IR injury is the anaphylatoxin C5a (4, 49, 57). C5a is a fragment of the activated complement factor C5 and has the ability to modulate the neutrophil response to injury and cause cell death through release of reactive oxygen species and granule-based enzymes (12, 20, 53). We found that MRL/lpr mice treated with C5aRA, a peptide that targets the C5a receptor and, thus, blocks the effects of the C5a complement fragment, are protected from local tissue damage after mesenteric IR compared with mice treated with control peptide (Fig. 7, A–D and I). Mucosal destruction, loss of villi and epithelial cells, and hemorrhage were significantly decreased in the

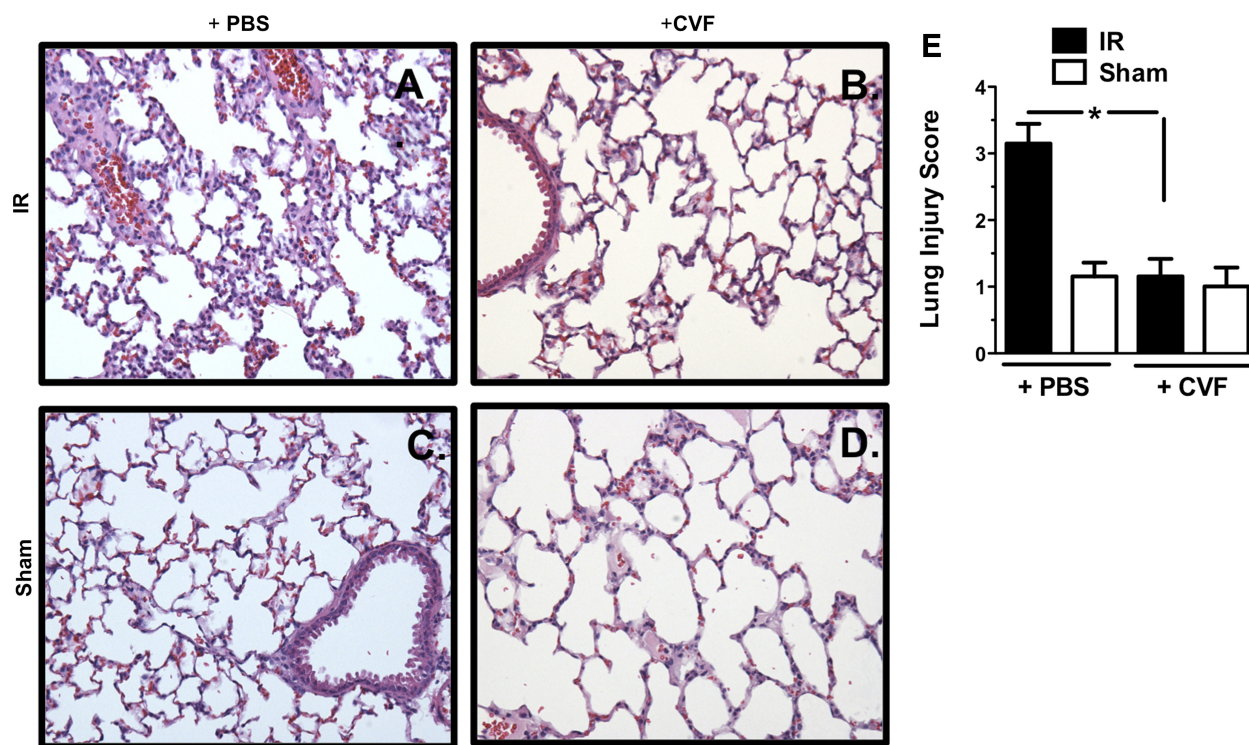


Fig. 5. Remote lung tissue damage is reduced following mesenteric IR in complement-depleted MRL/lpr mice. A–D: hematoxylin-eosin-stained sections of mouse lung tissues after 30 min of ischemia and 2 h of reperfusion. Images are representative of a total of 6 mice. Representative images are shown at  $\times 200$  magnification. E: injury score in intestine. Values are means  $\pm$  SD of a total of 6 mice for each control and experimental group in 3 separate experiments. \* $P \leq 0.05$ .



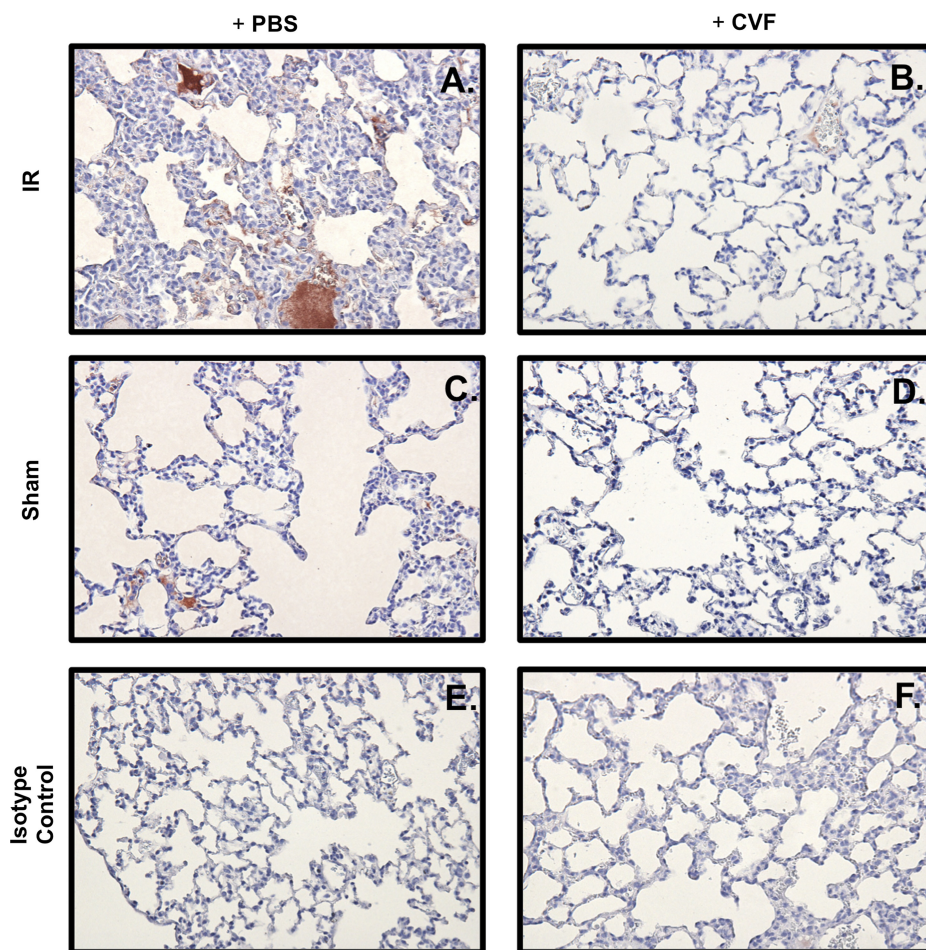


Fig. 6. C3 deposition is reduced in lung tissue following mesenteric IR in complement-depleted MRL/*lpr* mice. A–F: IHC with an anti-C3 antibody (red). All representative images are shown at  $\times 200$  magnification. Nuclei stained blue with hematoxylin. A total of 6 mice were used for each control and experimental group in 3 separate experiments.

C5aRA-treated mouse intestine after mesenteric IR injury compared with control mice. Blockage of the C5a receptor greatly reduced neutrophil recruitment in the intestine (Fig. 7, E–H) compared with control mice. In addition, differences in the levels of proinflammatory cytokines showed the same trend observed in the previous experiments, although they were not statistically significant (data not shown). Administration of C5aRA did not protect MRL/*lpr* mice from remote lung tissue damage (Fig. 8), indicating that additional factors contribute to the expression of remote lung tissue damage.

## DISCUSSION

In this study, we show, in a lupus mouse model, that complement depletion mitigates tissue damage after mesenteric IR. In particular, we demonstrate that intestinal tissue damage is significantly reduced in complement-depleted MRL/*lpr* compared with complement-sufficient MRL/*lpr* mice. The protection was linked with a decrease in C3 and C5b-C9 complex deposition, a reduction in mRNA levels of proinflammatory cytokines, decreased levels of oxidative stress, and a significant reduction in the infiltration of neutrophils in the damaged tissue. Next, we evaluated the effects of complement depletion in the development of remote tissue damage. Consistent with previous studies (11, 55), we showed that complement-depleted mice are also protected from remote lung damage after mesenteric IR. Noticeably, C3 deposition was significantly

reduced in lung tissue of complement-depleted mice. Our results establish that complement modulation can protect lupus-prone mice from tissue damage resulting from mesenteric IR injury (2, 16, 18, 25, 37, 48).

To our knowledge, the role of complement depletion in IR injury using a lupus mouse model has not been investigated until now. The MRL/*lpr* mouse is an autoimmune-prone mouse model that has been shown to present with significantly increased tissue damage after mesenteric IR compared with normal B6 mice (17). It has been claimed that ischemic cells express new surface antigens to which readily circulating autoantibodies can bind and, in turn, activate the complement cascade, creating a positive-feedback loop in the inflammatory response. However, it is important that a transient ischemic episode is needed for the expression of tissue damage, and the presence of these autoantibodies alone does not lead to tissue injury. The increased susceptibility of these mice to tissue damage after IR injury may reflect the sensitivity of SLE patients to tissue damage. We can speculate that comorbidities, such as atherosclerosis, immune-complex related renal damage, and various forms of vasculopathies, in SLE patients (36, 45, 47) can cause local reduction of blood flow, leading to a temporary ischemic state that could be enough to initiate the IR cascade. However, more studies are needed to confirm the contribution of IR-related damage in the clinical expression of SLE.



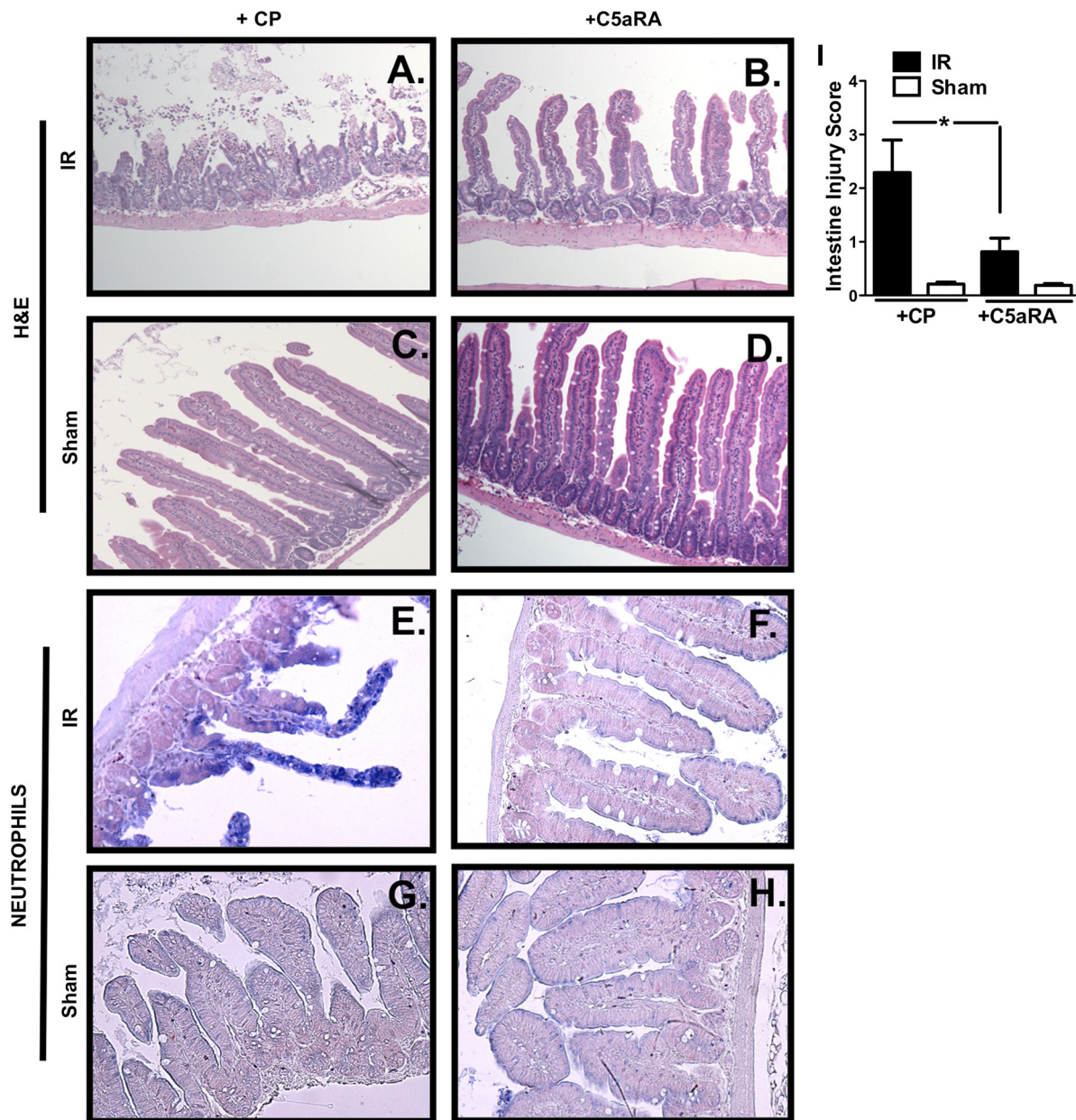


Fig. 7. Intestinal damage is reduced following mesenteric IR in MRL/*lpr* mice after administration of the C5a receptor antagonist. A–D: hematoxylin-eosin (H&E)-stained sections of mouse small intestine after 30 min of ischemia and 2 h of reperfusion. A total of 4 mice were used in 2 separate experiments. E–H: images of neutrophil staining (blue). All representative images are shown at  $\times 100$  magnification. I: injury score in intestine. CP, control peptide. Values are means  $\pm$  SD. \* $P \leq 0.05$ .

Complement activation leads to production of the C3a and C5a anaphylatoxins (50). The anaphylatoxin C5a has been implicated in the promotion and amplification of the inflammatory reaction (12, 20, 53), and other studies have demonstrated that C5a is a central player in the expression of tissue damage after IR (4, 19, 49, 57). Consistent with the above-mentioned studies, our treatment using a C5aRA in the MRL/*lpr* mouse model of IR was able to protect these mice from local tissue damage. A significant pathological improvement in tissue damage and a reduction in neutrophil infiltration in the intestine are demonstrated in the current study. However, blockage of the C5a receptor was not able to protect mice from remote lung tissue injury, indicating that more factors are involved in this process.

The difference between the CVF treatment and the C5aRA can be explained by the different mechanisms of action. CVF depletes all complement factors through activation; thus, by the time of the ischemic assault, no complement is available to initiate an immune response as a result of the damage. On the other hand, the C5aRA is administered after the ischemic injury and after complement activation; thus only a component of the complement cascade is blocked, which, as our results suggest, is beneficial for the local tissue damage, but not for the remote lung damage. This can also explain why the differences in the mRNA levels of the proinflammatory cytokines in the lung after treatment with C5aRA do not reach a statistically significant level (data not shown). We believe that a level of inflammatory response is still being induced, because other

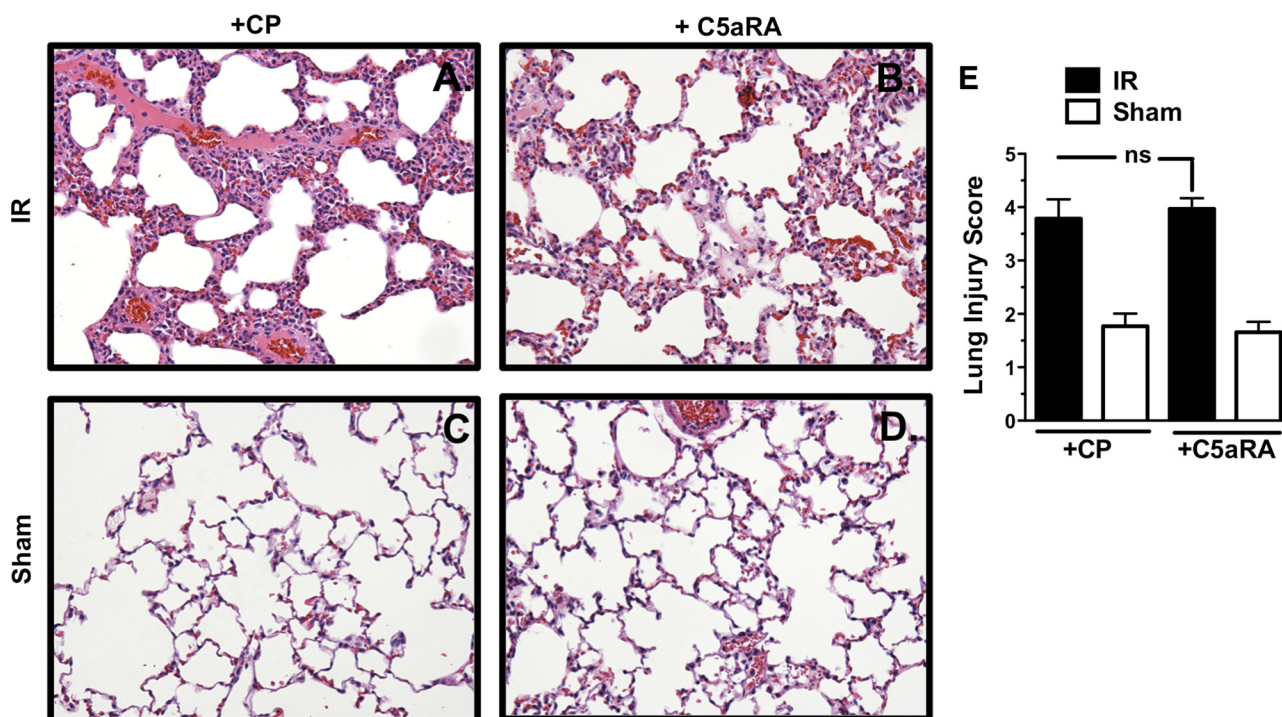


Fig. 8. Remote lung tissue damage is not reduced following mesenteric IR after administration of the C5a receptor antagonist. A–D: hematoxylin-eosin-stained sections of mouse lung tissues after 30 min of ischemia and 2 h of reperfusion. A total of 4 mice were used for each control and experimental group in 2 separate experiments. All representative images are shown at  $\times 200$  magnification. E: injury score in intestine. Values are means  $\pm$  SD. ns, Not significant.

fragments of activated complement factors, such as C3a or C4b, may lead to the activation of different cells such as platelets, resulting in the expression of remote lung damage. Recent studies from our laboratory and others have emphasized the importance of complement activation by platelets and vice versa (23, 35), as well as the novel role of platelet activation and platelet-derived molecules, in the expression of tissue damage in IR injury (29–32) and in autoimmune diseases (13, 14, 38).

In conclusion, we have shown that CVF can effectively deplete complement in lupus-prone mice and suppress the inflammatory response and injury in tissues experiencing IR injury and mitigate remote organ damage. The fact the blockage of the action of C5a protects only local injury but fails to limit remote organ damage suggests that other components of the activated complement cascade or blood cells traveling through the reperfused organ account for the development of remote organ damage.

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#### DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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